

## PLATELET SURVIVAL

### Introduction

The presence in the blood of small particles, probably platelets, was observed by Brown in 1861 and established by Hagerup in 1865. Hagerup also observed that the life span of platelets was very short and this has been confirmed by many workers. The life span of platelets was estimated indirectly by measuring the time for the platelet count to return to normal after bleeding (Gordon, 1925) and in man by observing the rate of fall of platelet count in thrombocytopenic patients (Hirsch and Gordon, 1951). Further indirect evidence came from study of the effects of, and the response to, irradiation of platelets. These studies have shown a life span of from 2 to 10 days.

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In the 1950s the introduction of radioisotopes gave a new impetus to the study of platelets. The measurement of the survival of platelets is now commonly used in the investigation of patients with thrombocytopenia. Survival studies over the liver and spleen, following the administration of radioactively labelled red cells, is known to be of value in the selection of such patients for splenectomy. Platelet survival using isotopes has been studied in several centres (Ans and Gordon, 1958; Davey and Lister, 1961) and there are few reports of the literature from this country (Field and Lister, 1966). There have been two main fields of interest, the thrombocytopenias (Wells, 1961; Gordon and Hammett, 1961; Mason, Ardill, Caen, Larrieu and Bernard, 1962) and leukaemia (Murphy and Gordon, 1962). The present work was undertaken to develop a method of estimating platelet survival in order to define more fully the role of the spleen in the production of thrombocytopenia, and to assess whether the measurement of platelet life span with isotopes could be used to predict the response to splenectomy of patients with idiopathic thrombocytopenic purpura. Platelet survival has also been studied in patients with increased



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### Introduction:

The presence in the blood of small particles, probably platelets, was observed by Donne in 1842 and established by Bizzozero in 1882 (quoted from Odell and Knisely, 1962). Indirect evidence suggested that the platelets were formed in the megakaryocytes of the bone marrow and this has been confirmed by bone marrow culture (Albrecht, 1957). Platelet life span in animals was estimated indirectly by measuring the time for the platelet count to return to normal after bleeding (Bedson, 1923) and in man by observing the effect of platelet transfusion into thrombocytopenic patients (Hirsch and Gardner, 1951). Further indirect evidence came from study of the effects of, and the recovery from, therapeutic or accidental irradiation. These studies suggested a platelet life span of from 2 to 10 days.

In the 1950s the introduction of radioisotopes gave a new impetus to the measurement of the survival of the cells of the blood. The measurement of the survival of red blood cells is now commonly used in the investigation of patients with haemolytic anaemia. Surface counting over the liver and spleen, following the administration of radioactively labelled red cells, is known to be of value in the selection of such patients for splenectomy. Platelet survival using isotopes has been studied in several centres (Aas and Gardner, 1958; Davey and Lander, 1963) but there are few reports in the literature from this country (Alfos, Field and Ledlie, 1959). There have been two main fields of interest, the thrombocytopenias (Cohen, Gardner and Barnett, 1961; Najeon, Ardaillou, Caen, Larrieu and Bernard, 1963) and atheroma (Murphy and Mustard, 1962). The present work was undertaken to develop a method of estimating platelet survival in order to define more closely the role of the spleen in the production of thrombocytopenia, and to assess whether the measurement of platelet life span with surface counting could be used to predict the response to splenectomy of patients with idiopathic thrombocytopenic purpura. Platelet survival was also studied in patients with increased

platelet counts.

#### Methods:

Two platelet labels have been used, sodium chromate with incorporated  $^{51}\text{Cr}$  and diisopropylphosphoro-fluoridate (DFP) in which the phosphorus is  $^{32}\text{P}$ . Some of the advantages and disadvantages of these two methods are outlined in Table 1.

#### Sodium Chromate Method:

We have used a modification of the method proposed by Aas and Gardner (1958) for the in vitro labelling of platelets with sodium chromate. One pint of blood is collected from the patient or from an ABO Rh compatible donor into a Fenwal plastic pack in which the anticoagulant is disodiummethylenediamine tetracetic dihydrate (EDTA) in saline. The whole blood is centrifuged at  $2^{\circ}\text{C}$  for 20 minutes, at 1000 r.p.m. in an MSE Major centrifuge. The platelet rich plasma is then transferred with a plasma extractor to a sterile siliconed MRC glass bottle which is spun at 2000 r.p.m. for 15 minutes. The plasma is withdrawn and the platelet "button" resuspended in 8-10 ml. of normal saline. 250 - 400  $\mu\text{c}$ . of sodium  $^{51}\text{Cr}$  chromate are added and the mixture incubated at  $25^{\circ}\text{C}$  for one hour. Approximately 80 ml. of normal saline are added and the mixture centrifuged at  $4^{\circ}\text{C}$  for 10 minutes at 2000 r.p.m. The supernatant is kept for measurement of its radioactivity. The platelet "button" is again resuspended in normal saline, to a volume of about 20 ml. A small aliquot is retained for measurement of its radioactivity and the remainder is given by intravenous injection to the patient to be studied. The amount of radioactivity varies from 20-100  $\mu\text{c}$ .

#### Comments on Labelling Procedure:

1. Equipment: Plastic packs and siliconed glassware are used to minimise the loss of platelets (Aas and Gardner, 1958; Najean et al, 1963). The recovery of platelets from the original pint of blood varied from 30 to 80% with an average of 60%. Higher values than this were achieved at the expense of greater contamination with red cells.

2. Anticoagulant: The anticoagulant used was EDTA as most workers have shown a higher recovery of platelets with this, than when using the normal acid-citrate-dextrose (ACD) mixture as anticoagulant. It now appears that a preferable anticoagulant would be an acid-citrate-dextrose mixture with a higher concentration of citrate as proposed by Aster and Jandl (1964). Platelets suspended in EDTA are more spherical and clump less readily than with the normal ACD. But EDTA may damage platelets which are naturally more disc-shaped. The increased acidity of the citrate-enriched ACD mixture apparently allows the platelets to assume their normal disc shape without rendering them unduly "sticky". The effect is a greater yield of platelets from the patient and much less sequestration. The present work was continued with EDTA to allow all the results to be comparable.

3. Donor platelets: It is known that the platelets share the A and B antigens of the erythrocytes (Dausset, 1963). In all cases when donor platelets were used, the platelets were obtained from ABO Rh compatible blood. This does not exclude incompatibility due to other antigens but in no case was unexpected shortening of the platelet lifespan found except in two patients who had recently had multiple transfusions.

4. Time of Incubation: We have found that when incubating at  $25^{\circ}\text{C}$  the uptake of sodium <sup>51</sup>chromate increases up to 90 minutes (Fig. 1) and then diminishes. This agrees with the findings of Davey and Lander (1963) who found a linear increase with time (at  $24^{\circ}\text{C}$ ) up to 40 minutes but disagrees with the observations of Aas and Gardner (1958) who found no increase after 15 minutes.

5. Temperature of Incubation: The uptake of chromate by platelets incubated for 30 minutes increases as the temperature is raised to  $37^{\circ}\text{C}$  but falls thereafter (Fig. 2). Davey and Lander (1963) also reported increasing labelling efficiency up to  $37^{\circ}\text{C}$  but Aas and Gardner (1958) noted a decrease between room temperature ( $20-22^{\circ}\text{C}$ ) and  $37^{\circ}\text{C}$ .

6. Chromium Concentration: Concentrations of chromium above

3  $\mu$ gm. per  $10^{10}$  platelets diminishes labelling (Fig. 3). This agrees with the findings of other workers.

7. Washing of Labelled Platelets: Initially this was done but the supernatant wash always contained less than 5% of the radioactivity bound to the platelets and usually less than 3%. Thereafter washing was not performed but the addition of 80 ml. of normal saline to the incubating mixture before the final centrifugation of the platelet button allowed removal of more than 99% of the radioactivity not bound firmly to the platelets.

8. Superinone: Initially, this compound, also known as Triton or WR-1339, was added to the saline used for suspension and washing of the platelets. It is a non-toxic, surface active substance believed to render platelets less sticky and thus facilitate re-suspension of platelets. In practice its use was found to be unnecessary.

9. Efficiency of labelling: The uptake of sodium  $^{51}\text{Cr}$  chromate by the platelet fraction from one pint of blood varied from 12.5 to 40%. The higher values are obtained with high platelet yield and the lower efficiencies are seen when the platelet recovery is poor, when the  $^{51}\text{Cr}$  has a low specific activity, leading to a high concentration of chromate, or when the platelet fraction is contaminated with red cells, which have a much greater affinity for chromate.

10. Determination of Platelet Bound Radioactivity: Two 1 ml. aliquots of the platelet infusion are taken. One is diluted in 200 ml. water. The other is washed in 1% ammonium oxalate which lyses contaminating red cells, thus releasing the haemoglobin-bound sodium  $^{51}\text{Cr}$  chromate into the supernatant. Thus the total radioactivity injected may be calculated and also the proportion of that radioactivity which is bound to platelets.

11. Site of Binding of Chromate to Platelets: This is unknown.

#### DFP Method:

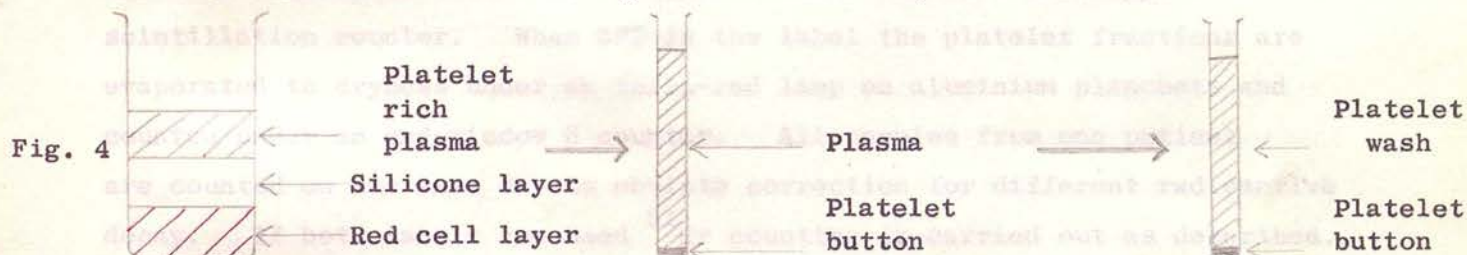
We have used this toxic compound in a dose of 100  $\mu$ c. given by intravenous injection. It is obtained from Amersham dissolved in propylene glycol, with a high specific activity so that this amount of radioactivity is contained in an amount of the compound which is well below the toxic level



to the body. There were no untoward reactions to the substance. DFP binds irreversibly to esterases in the cell membrane and is a satisfactory in vivo label for red cells (Cohen and Warringa, 1954) and platelets (Leedsma and Cohen, 1956; Murphy and Mustard, 1962). Its main disadvantage is that the amount of red cell binding is much greater than platelet binding, necessitating careful separation of the platelets for counting. The amount of platelet bound radioactivity is too small to count in patients with severe thrombocytopenia.

#### Preparation and Counting of Blood samples:

Following labelling of the platelets, blood samples are taken one hour after infusion of the labelled platelets or DFP and daily thereafter for up to 12 days. The volume initially was 20 ml. but latterly 10 - 15 ml. has been found to be adequate. The anticoagulant used is 2% EDTA. The platelets are separated by a modification of the silicone flotation technique of Morgan and Szafir (1961). Approximately 5 ml. of silicone mixture (85% MS 555 Hopkin and Williams and 15% MS 200/20 CS Hopkin and Williams) are added to the mixture of whole blood and EDTA, in a 50 ml. siliconed centrifuge tube. This is centrifuged at 1200 r.p.m. for 10 minutes in an MSE Major Refrigerated centrifuge. The specific gravity of the silicone mixture is 1.040 which is between the specific gravity of platelets and red blood cells. The centrifugation leads to separation of the red cells



and platelets (Fig. 4). The platelet rich plasma also contains the lighter red cells which are reticulocytes and young cells, a few polymorphs and many lymphocytes.  $^{51}\text{Cr}$  labels red cells which are thus largely removed.  $\text{DF}^{32}\text{P}$  labels polymorphs and red cells which are largely removed. Lymphocytes are not significantly labelled by DFP. The platelet rich plasma is pipetted into a clean siliconed 10 ml. centrifuge tube which is spun at 3000 r.p.m. for 10 minutes. The supernatant plasma is removed and the platelet

button resuspended in 1% ammonium oxalate. The tube is again centrifuged at 3000 r.p.m. for 10 minutes, the supernatant discarded and the final platelet button resuspended in 3 ml. ammonium oxalate ready for counting if chromate is the label and ready for evaporation on to a planchet if the label is phosphoro-fluoridate. Contaminant red cells are lysed by the wash in ammonium oxalate which also removes most of the polymorphs not already separated. Further washing with 1% ammonium oxalate can be done. This is not necessary for  $^{51}\text{Cr}$  as the method used gives fairly high radioactive counts in the platelet fraction and little in the red cells but is usually required (three washes) when DFP has been used in order to obtain complete separation of the platelets from the red cells (which have a greater affinity for DFP). The above method gives a 100% yield of platelets. The figures found vary from 90 - 105% which is within the experimental error of counting (Dacie and Lewis, 1963). Many workers have related radioactive counts in the platelets to the actual number of platelets. Using the above method we have found that this is unnecessary and instead count the amount of radioactivity in the platelet fraction of a known volume of whole blood. We believe that the error of counting platelets (at least 10%) is greater than the error of separation of platelets by this method.

Samples with  $^{51}\text{Cr}$  are counted in a standard well type scintillation counter. When DFP is the label the platelet fractions are evaporated to dryness under an infra-red lamp on aluminium planchets and counted under an end-window  $\beta$  counter. All samples from one patient are counted on the same day to obviate correction for different radioactive decay. If both labels are used  $^{51}\text{Cr}$  counting is carried out as described. The samples are then dried on planchets and their  $\beta$  counts determined. This count is corrected for activity due to  $^{51}\text{Cr}$  in order to obtain the "true" DFP count. Counting is carried out to allow an error of less than 3%.

#### Surface Counting:

This is carried out daily over the praecordium, liver and

spleen using a directional scintillation counter with fairly wide collimation. Counting is done for 3 - 10 minutes according to the rate of the count. In a few patients two matched scintillation counters connected to ratemeters and recorders have been used to give a continuous record immediately following infusion of the  $^{51}\text{Cr}$  labelled platelets. The sites for surface counting are as follows: praecordium - fourth left intercostal space at sternal edge; liver - ninth right intercostal space half-way between the anterior and mid-axillary lines; spleen - no standard position has been used. The one adopted is that which gives a maximum count for the patient. In patients with idiopathic thrombocytopenia for example, in whom the spleen is normal in size, the counter will be sited over the 10th-11th left intercostal space on the posterior axillary line. In patients with splenomegaly the positioning need not be as precise and is usually over the costal margin on the left anterior axillary line.

#### Interpretation of Results:

The maximum radioactivity in any platelet fraction of a patient is taken as 100% and the radioactivity of the other samples expressed as percentages of the maximum. This allows comparison of all results. Platelet lifespan is expressed as lifespan or extinction time, and is the time in days when the platelet-bound radioactivity is less than 5% of the maximum. The  $T_{\frac{1}{2}}\text{Cr}$  or  $T_{\frac{1}{2}}\text{DFP}$  is not used because of doubt as to the exact shape of the survival curves.

In the interpretation of surface counting data in studies with  $\text{Na}_2^{51}\text{CrO}_4$  labelled red cells two factors are commonly accepted as important (Jandl, Greenberg, Yonemoto and Castle, 1956). The splenic sequestration index is the percentage increment in the spleen/praecordium ratio between the start of the survival study and the  $T_{\frac{1}{2}}\text{Cr}$ . (The  $T_{\frac{1}{2}}\text{Cr}$  is the time for half of the radioactivity to disappear from the blood). This value is not valid in platelet survival studies because of the early irreversible sequestration of some platelets in the spleen. The other index used is the spleen/liver ratio at  $T_{\frac{1}{2}}\text{Cr}$  but the value of this is also questionable because of the sequestration noted above. The spleen/liver ratio and the splenic



sequestration index are easy to use because correction of the counts for radioactive decay is unnecessary. The spleen/liver ratio has been calculated in our patients.

In an attempt to standardise the results for all patients and to assess the amount of radioactivity in the spleen, the surface counts over the spleen at  $T_{1/2}Cr$  have been corrected for decay and divided by the total amount of injected platelet-bound radioactivity in micro-curies. This is an arbitrary value, the results of which depend inter alia on the counting system used. The present one in this study gives a count of 18 counts/second/micro-curie at a distance of 10 cm. from the crystal, with a background of 11 counts/second. A similar value has been used by Aster and Jandl (1964). The determination of injected platelet-bound radioactivity is discussed above (Sodium Chromate Method - Comment 10).

## Results:

### Normal Platelet Survival

In Fig. 5 are shown the survival curves of  $^{51}Cr$  labelled platelets in two normal patients. On the left is illustrated the relative amounts of radioactivity in the component fractions of the blood samples. Usually there are insignificant counts in the red cells or plasma. However if the original platelet infusion is contaminated with red cells (which will also be labelled) the amount of activity in the red cells becomes very significant as the platelet activity falls. Therefore platelet fractions are counted and not whole blood.

The radioactivity in the platelets falls in a curvilinear fashion and becomes negligible (less than 5% of the maximum) at 10 days, the so-called extinction time. Eight normal subjects have had platelet survival times of 8-12 days. Autologous and isologous platelets give similar results although we have no figures of direct comparison.

For ease of comparing results the maximum platelet activity is taken as 100% and all other samples expressed as a percentage of this. This is demonstrated on the right of Fig. 5, in which the results from

another control subject are also seen. The second survival curve (J.M.) shows that the platelet activity is less one hour after infusion than after one day. This is believed to be due to sequestration of a large proportion of the injected platelets in the first 30 minutes after infusion (Davey and Lander, 1964; Aster and Jandl, 1964). A variable proportion of these sequestered platelets return to the circulation within 24 hours. Davey and Lander (1964) have made a most extensive study of the initial sequestration and believe that it occurs throughout the vascular bed but particularly in the liver, spleen and lungs. Surface counting by us has confirmed the importance of the liver and spleen as sites for sequestration but we have not observed significant counts over the lungs.

Studies using  $DF^{32}P$  gives very similar results to  $^{51}Cr$ . The survival curve is identical but occasionally the latter part of the curve shows some "tailing" (Fig. 6). This has been noted by Barkham (1966) and may be due to labelling of plasma proteins not removed by washing or to partial re-utilisation of the label. Platelet lifespan with DFP is also 8-12 days.

#### Comparison of Labels:

$DF^{32}P$  and  $^{51}Cr$  have been directly and simultaneously compared in 15 patients, 3 normal subjects, one patient with carcinoid syndrome, 2 patients with spherocytosis, one patient with essential thrombocythaemia, 3 with reticuloses, 3 with cirrhosis of the liver and portal hypertension, and 2 patients with idiopathic thrombocytopenic purpura following splenectomy. The graphs of the survival of the platelets of 8 patients are shown in Fig. 6. There is excellent correlation. In some patients there is a tendency to a "tail" in the  $DF^{32}P$  curves. Most of the  $^{51}Cr$  curves show more radioactivity on day 1 than 1 hour after infusion of the labelled platelets.

On one occasion we have found a discrepancy between the results given by the two methods (Fig. 7). In this man with idiopathic thrombocytopenia before splenectomy the platelet count was 20,000/cu.mm. and the platelet survival, estimated with  $^{51}Cr$  labelled donor platelets, was markedly shortened to 1 day. Following operation the platelet count

returned to normal and as expected, the survival of the patient's own platelets, labelled with DFP, was normal. However  $^{51}\text{Cr}$  labelled donor platelets showed continued shortening of platelet lifespan. This may have been due to iso-immunisation resulting from blood transfusion at time of operation or from the previous platelet infusion.

#### Idiopathic Thrombocytopenic Purpura (ITP)

Seventeen patients have been studied. Four had failed to respond to splenectomy and had persisting thrombocytopenia. Eleven patients were studied prior to splenectomy and six of them proceeded to splenectomy, which led to a remission in all. Platelet survival was also studied in two patients whose spleens had been removed some years before and who were in remission with normal platelet counts. The results are summarised in Table 2. In all donor platelet labelled with  $^{51}\text{Cr}$  were used.

In three patients with thrombocytopenia and megakaryocyte hyperplasia in the marrow, the platelet survival was normal. These patients are of particular interest. In all other patients with thrombocytopenia pre- or post-splenectomy, the platelet survival was markedly reduced. Six of the patients with thrombocytopenia studied, proceeded to splenectomy and in all the platelet count returned to normal; in three the platelet survival was also measured after splenectomy and was normal. In all patients with normal platelet counts the platelet survival was normal.

#### Thrombocythaemia and Thrombocytosis

We have measured platelet survival in five patients who had increased platelet counts in the peripheral blood. In all the lifespan of the platelets was normal (Fig. 8). One patient had essential thrombocythaemia and four had thrombocytosis following removal of the spleen for thrombocytopenia due to disseminated lupus erythematosus, spherocytosis (two patients) and traumatic rupture of the spleen.

#### Hypersplenism

Eighteen patients with large spleens and low white blood cell counts and platelet counts have been studied. The results and diagnoses

are given in Table 3. The platelet survival was normal in all but two patients who had lymphoproliferative disorders.

#### Aplastic Anaemia

Two patients with this condition were studied and both had normal platelet lifespan.

#### Surface Counting

In the section on methods the interpretation of surface counting data is discussed. In Fig. 9 the data on patients with idiopathic thrombocytopenic purpura and hypersplenism and in normal subjects, are presented. The spleen/liver ratios in patients with ITP (six of whom subsequently had splenectomies performed with beneficial results) are not different from normal patients. Patients with hypersplenism have values which overlap normals but which are mostly higher. The calculated absolute spleen counts in patients with ITP are greater, relative to the infused radioactivity, than in patients with hypersplenism or normal subjects. It should be pointed out that the total radioactivity in the spleen in patients with splenomegaly will be relatively greater than in normal subjects or patients with ITP, as one is counting over a smaller proportion of the spleen in patients with splenomegaly.

The actual liver counts/injected radioactivity are of course also higher in patients with ITP, and it is evident from these results that a greater proportion of the platelets infused are destroyed in the liver and spleen in patients with ITP than in normal subjects in whom the platelets may be destroyed throughout the vascular bed.

In Fig. 10 are shown the results of surface counting and platelet lifespan measurements in 9 patients who had been subjected to splenectomy before study. Four had ITP and were "failed splenectomy", two had responded to removal of the spleen and three had the spleen removed for other reasons - spherocytosis, traumatic rupture and portal hypertension. Patients with ITP failing to respond to splenectomy, have a reduced survival of platelets associated with a high uptake by the liver.



### Tissue Measurements of Radioactivity

Two patients with cirrhosis, portal hypertension and splenomegaly died shortly after completion of platelet survival studies. The tissues of the body were analysed for radioactivity. There were small amounts of activity in all tissues but significant amounts only in the spleen, the liver and the bone marrow.

Surface counts over the spleen and liver were compared with tissue counts in several patients with ITP who proceeded to splenectomy at which liver and splenic biopsies were obtained. The work of Aster and Jandl (1964) was confirmed in showing that the efficiency of counting over the spleen is four times that over the liver when the surface counts are compared with counts per gramme of tissue.

### Discussion:

#### Methods

The method for labelling of platelets with  $^{51}\text{Cr}$  involves several compromises. We have chosen  $25^{\circ}\text{C}$  and 60 minutes incubation time as values which allow adequate labelling but which do not cause damage to the platelets. The method of labelling with  $\text{DF}^{32}\text{P}$  is a standard one but we believe that the separation of platelets from blood samples by silicone flotation followed by washing in 1% ammonium oxalate allows for optimum recovery of platelets and platelet-bound radioactivity and obviates platelet counting of separated fractions, which is both tedious and inaccurate. The unsatisfactory points of the methods are the extent of sequestration with  $^{51}\text{Cr}$  and the unsuitability of  $\text{DF}^{32}\text{P}$  as a label in patients with severe thrombocytopenia. The sequestration is due to alteration of the properties of the platelets by the anticoagulant used, EDTA, perhaps by removal of magnesium  $\text{Mg}^{++}$  ions by chelation (Aster and Jandl, 1964). Further experience is intended with a modification of the usual acid-citrate-dextrose mixture as anticoagulant. It may be possible to use  $\text{DF}^{32}\text{P}$  in patients with platelet counts down to 30,000/cu.mm. if counting techniques can be improved, perhaps by the use of liquid scintillation counting.

We have little experience as to the extent of elution of the two labels from the platelets. However, we feel that if elution were an important factor, it would be unlikely to be identical with the two labels in which event there would not be good correlation between the two methods. Aas and Gardner (1958) found 40-70% elution in one hour from platelets labelled with  $\text{Na}_2^{51}\text{CrO}_4$  and then incubated at room temperature in vitro, but believed that elution did not take place in vivo. We have not observed a significant leak of  $^{51}\text{Cr}$  from platelets labelled as described, and subsequently left at room temperature for up to 90 minutes after completion of preparation of the labelled platelets.

Apart from the difficulty of interpretation of surface counting data following infusion of  $^{51}\text{Cr}$  labelled platelets from blood anticoagulated with EDTA, the recovery of platelets in the peripheral blood is only 10 - 60% of that injected, due also to sequestration of platelets. Aster and Jandl using EDTA obtained recoveries of only 5 to 30% but when a modified ACD anticoagulant was used, the recovery of platelets increased to 75%.

#### Normal Survival

There has been considerable discussion as to the precise nature of the survival curves. Most of our survival curves are almost linear but a slight deviation from the straight line is evident in some. This curvilinear type of graph is in favour of death by senescence rather than the alternative theory of random destruction of platelets which would give an exponential type of curve. The problem has been studied in considerable detail, using  $\text{DF}^{32}\text{P}$ , by Mustard, Rowsell and Murphy (1966) who have proposed a "multiple-hit" hypothesis, in which the lifespan of an individual platelet depends on the number of "insults" it sustains. If they are few the platelet will survive "normally" to 10 days; if they are many it will be used up in a day or two. The truth may be that most platelets normally "die" by senescence, and are thus directly comparable to red blood cells, but some may be destroyed earlier by virtue of their function such as maintaining the integrity of the endothelial surfaces. Mustard, Rowsell and Murphy (1966) have found in pigs that when the platelet

count is low, platelets infused will have a lifespan shorter than normal and this would support their "multiple-hit" hypothesis. But in two patients with aplastic anaemia and three patients with apparent idiopathic thrombocytopenic purpura, we have found a normal survival of infused platelets when the platelet count was very low indeed. Many patients with hypersplenism have normal platelet survival and thrombocytopenia. Therefore we do not believe that survival of platelets is largely dependent on the platelet count in the peripheral blood.

#### Idiopathic Thrombocytopenic Purpura

This condition is diagnosed by exclusion of other causes of thrombocytopenia, in the presence of a reduced platelet count and megakaryocytic hyperplasia in the marrow. It has been suggested that it is an autoimmune condition in which the platelets are prematurely destroyed by antibodies, which are possibly produced in the spleen in cases responding to splenectomy. Techniques for demonstrating anti-platelet antibodies are technically very difficult and in the best hands give positive results in less than 50% of cases (Dausset, 1963; Cohen, Gardner and Barnett, 1961). If the autoimmune hypothesis is correct the thrombocytopenia is due to shortened platelet lifespan. This has been demonstrated by several workers (Aas and Gardner, 1958; Cohen, Gardner and Barnett, 1961; Najean, Ardaillou, Caen, Larrieu and Bernard, 1963; Aster and Jandl, 1964). We have shown reduction of donor platelet lifespan in patients with idiopathic thrombocytopenic purpura and a return to normal after splenectomy. Because of the normal platelet survival found in patients with aplastic anaemia in whom there was as marked thrombocytopenia, we do not believe that the shortened platelet lifespan in patients with ITP is due to increased utilisation of platelets. If the platelets are prematurely destroyed in ITP following an immune reaction, then one would expect the survival curve to be exponential in nature as is seen with the red cells in acute autoimmune haemolytic anaemia. Unfortunately due to the initial partially reversible sequestration of platelets following infusion, the shape of the survival curve in the first day is extremely difficult to interpret and the platelet lifespan may be only 1 - 2 days. Present

techniques do not permit the use of  $DF^{32}P$  to elucidate the nature of the survival curve as there is insufficient radioactivity in the platelet fractions of the blood.

#### Thrombocythaemia and Thrombocytosis

We have not found a prolonged platelet survival in any patient with an increased platelet count whether  $^{51}Cr$  or  $DF^{32}P$  is used as the platelet label. Aster and Jandl (1964) reported normal survival of platelets labelled with  $^{51}Cr$  in a patient with essential thrombocythaemia. However Alfos, Ledlie and Field (1959) using  $DF^{32}P$  claimed to show prolonged platelet lifespans in 3 patients with essential thrombocythaemia. We believe that the increased platelet count in patients with thrombocythaemia or post-splenectomy thrombocytosis is due to increased production and not prolonged platelet survival.

#### Hypersplenism

Cohen, Gardner and Barnett (1961) have reported normal platelet survivals in patients with hypersplenism. We have confirmed this except in two cases with lymphoproliferative disorders in which an alteration in the immune reactions of the lymphocytes might be expected to lead to the production of abnormal antibodies against the platelets. It is known that in these conditions autoimmune haemolytic anaemia may occur. The cause of the thrombocytopenia in patients with splenomegaly and normal platelet lifespans is not known. It has been postulated that the spleen, through a humoral factor, may inhibit marrow production. But no agent has been demonstrated and the marrow in our patients showed normal numbers of megakaryocytes, confirming the report by Bouma, Veeger, Jansz, Woldring, Abels and Nieweg (1963). Recently it has been established that anaemia in patients with large spleens may in part be due to red cell pooling in the spleen (Toghill, 1964; Richmond and Donaldson, 1966). It is also known that in hypothermia reversible sequestration of platelets may occur in the liver and spleen (Bouma et al, 1963). Therefore it is postulated that the reduced platelet count in patients with splenomegaly and normal platelet survival is due to sequestration of a proportion of the platelets in the



spleen. This is supported by the work of Penny, Rozenberg and Firkin (1966) who, by perfusion of spleens removed surgically, demonstrated a pool of splenic platelets, the extent of which appeared dependent on spleen size alone. Our surface counting data showing high spleen/liver ratios also support this concept, and the ratios observed are artificially low as one is counting over a smaller proportion of the total splenic area in patients with splenomegaly, as opposed to normal subjects and patients with ITP.

The cause of the splenomegaly appears to be immaterial. We have observed normal platelet survival, like Cohen et al (1961) in patients with cirrhosis, reticulosis and leukaemia. It has also been found in two patients with idiopathic tropical splenomegaly and one patient with kala-azar, and this is the first report of platelet lifespan measurements in these conditions.

#### Surface Counting

The initial rapid sequestration of platelets labelled with <sup>51</sup>Cr in the liver and spleen masks subsequent changes in the radioactivity of these organs and makes the interpretation of surface counting over them difficult, particularly in ITP when the platelet survival may be only 24 hours. However, Najean et al (1963) reported that splenic localisation was uncommon in patients with idiopathic thrombocytopenic purpura, but when present, was associated with a good result from splenectomy. Patients not showing such localisation might or might not do well following removal of the spleen. Our data shows that although the spleen/liver ratio does not distinguish between normal subjects and patients with ITP, the absolute counts over the spleen, standardised for each patient, are higher than normal in such patients. Thus far all the patients studied before splenectomy have done well after operation and further experience is necessary before we shall know whether we shall be able to predict the response to operation. However the results do suggest that in patients with ITP a greater proportion of injected platelets is taken up by the spleen (and liver)

than in normals.

Our data on patients with ITP who had failed to respond to splenectomy indicates that the continued thrombocytopenia in these patients may be due to increased destruction in the liver.

As stated above the findings in patients with splenomegaly support the concept of a splenic pool of platelets.

#### Hypersplenism or ITP?

There were three patients studied in whom the results were particularly difficult to evaluate. They were three females of greatly differing ages who had clinical ITP, but had normal platelet lifespan and low spleen/liver ratios and low spleen counts. (See Table 2 and Fig. 9). The platelet counts were low and ranged from 10,000 to 40,000. Cohen et al (1961) reported four such cases and Aster and Jandl (1964) a further one. They reported normal megakaryocytes in the marrow but postulated a production deficit. However while this remains a possibility, all of our patients had normal or increased megakaryocytes in the marrow. Surface counting did not reveal any site of sequestration, and spleen counts were low. The condition is therefore not a form of hypersplenism. It is possible that infused platelets were treated differently from naturally produced platelets due to different antigenic composition, but this seems unlikely. If production is normal, and infused platelets survive normally, it is possible that the thrombocytopenia is due to a deficit in the platelets of the patient. We have a ready comparison in the red cells in the condition of spherocytosis. Further studies with DFP or autologous platelets labelled with <sup>51</sup>Cr are necessary before this condition may be ascribed more definitely to a deficit in production. A further possibility is that antibodies act only against the megakaryocytes thus causing a deficit in production or release, but this is not known to happen in the red cell series and can only be another hypothesis. Although gamma globulin has been shown to coat megakaryocytes in patients with ITP, in those patients' sera anti-platelet antibodies were demonstrated and reduced platelet survival may be inferred.

# Classification of Thrombocytopenias

Finally in Table 4 a tentative classification of the thrombocytopenias is shown. It resembles that proposed by Cohen et al (1961). It may need revision and extension in the light of future investigations.

It is well known that the liver, spleen and bone marrow are involved in the production of various clotting factors.

Essential thrombocythemia is a myeloproliferative disease characterized by an increased number of platelets in the blood. The disease is associated with normal platelet function. The increased platelet count is due to an increased production of platelets and not to prolonged platelet lifespan.

The thrombocytopenia of hypersplenism is usually associated with portal hypertension and increased spleen counts over the spleen. The concept that this type of thrombocytopenia is due to a splenic sequestration of platelets is attractive. The size of which is proportional to the extent of splenic tissue. The patients with hypersplenism who have splenic infarction and splenic abscesses are usually associated with portal hypertension and splenic infarction. In the patients with large spleen and reduced platelet survival the disease causing the hypersplenism is a myeloproliferative one in which abnormal antibodies might be produced.

In patients with splenic masses the platelet survival is normal.

Patients with idiopathic thrombocytopenic purpura (ITP) commonly show a marked reduction in platelet lifespan, in keeping with the hypothesis that the disease has an autoimmune origin and the thrombocytopenia is due to antibody-mediated destruction. There is a small group of patients with ITP in whom the platelet survival is normal. Presumably a defect in production of platelets has been suggested as the cause in these patients, but in view of the normal or increased numbers of megakaryocytes in the marrow, alternative mechanisms for the production of thrombocytopenia are suggested. Surface counting data suggests that in patients with ITP a greater proportion of large platelets is taken up by the spleen than in normal subjects and this has been shown in patients who have been splenectomized.

Patients with ITP who had failed to respond to splenectomy

## PLATELET SURVIVAL - SUMMARY

Methods are described for the in vitro labelling of autologous or isologous platelets with sodium <sup>51</sup>chromate and the in vivo labelling of platelets with di-isopropyl <sup>32</sup>phosphorofluoridate. Excellent correlation has been found in simultaneous application of the two methods. The technique used for surface counting over the liver, spleen and praecordium is described and the interpretation of surface counting data discussed.

Essential thrombocythaemia and post-splenectomy thrombocytosis are associated with normal platelet survival. The increased platelet count in those conditions is therefore ascribed to increased production of platelets and not to prolonged platelet lifespan.

The thrombocytoepnia of hypersplenism is usually associated with normal platelet survival and increased surface counts over the spleen. The results support the concept that this type of thrombocytopenia is due to sequestration of platelets in a splenic platelet pool, the size of which is dependent on the amount of splenic tissue. The patients with hypersplenism who were studied include three patients with tropical splenomegaly. In two patients with large spleens and reduction in platelet survival the disease causing the splenomegaly was a lymphoproliferative one in which abnormal antibodies might be produced.

In patients with aplastic anaemia the platelet survival is normal.

Patients with idiopathic thrombocytopenic purpura (ITP) commonly show a marked reduction in platelet lifespan, in keeping with the hypothesis that this disease has an autoimmune aetiology and the thrombocytopenia is due to anti-platelet antibodies. There is a small group of patients with clinical ITP in whom the platelet survival is normal. Previously a deficit in production of platelets has been suggested as the cause in these patients, but in view of the normal or increased numbers of megakaryocytes in the marrow, alternative mechanisms for the production of thrombocytopenia are discussed. Surface counting data suggests that in patients with ITP a greater proportion of injected platelets is taken up by the spleen than in normal subjects, and thus far such data has been found in patients who have responded to splenectomy.

In patients with ITP who had failed to respond to splenectomy



shortened platelet lifespan has been demonstrated, associated with a high uptake of injected platelets by the liver. In patients with ITP who had remissions after splenectomy or who had their spleens removed for other reasons, normal platelet lifespan has been found associated with a smaller uptake of injected platelets by the liver.

In the light of these findings a revised classification of the thrombocytopenias is suggested.

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# COMPARISON OF TWO PLATELET LABELS

$^{51}\text{CrO}_4$

Chromate (Sodium Chromate)

Chromate (Sodium Chromate)

Label in vivo

Label in vitro necessary

Indicator

$\gamma$ -emitter; allows surface counting

Preparation and counting of samples difficult

Preparation and counting of samples easy

Can be used in vitro and in vivo

Allows use of donor platelets

Not toxic

Relatively expensive

Expensive

Table 1

COMPARISON OF TWO PLATELET LABELS

$DF^{32}P$ (Di-isopropylphosphorofluoridate)	$Na_2^{51}CrO_4$ (Sodium Chromate)
Labels in vivo	In vitro labelling necessary
$\beta$ -emitter	$\gamma$ -emitter: allows surface counting
Preparation and counting of samples difficult	Preparation and counting of samples easy
Not possible in severe thrombocytopenia	Allows use of donor platelets
Toxic	Not toxic
Simultaneously can be used as red cell label	-
Relatively expensive	Inexpensive

Table 2

## STUDIES IN PATIENTS WITH I.T.P.

Patient	Sex	Age	Platelet count/cu.mm.	Post-splenectomy	Platelet lifespan (days)
S.C.	F	26	30,000	No	4
W.W.	M	70	15,000	No	1
R.S.	M	14	35,000	No	2
E.S.	F	18	<10,000	No	2
J.R.	F	36	<10,000	No	2
R.N.*	M	13	160,000	No	9
J.E.	M	10	<10,000	No	3
W.B.	M	68	20,000	No	5
A.A.	F	38	40,000	No	10
M.B.	F	78	<10,000	No	10
M.P.	F	7	30,000	No	10
J.A.	M	18	<10,000	Yes	1
S.J.	M	50	40,000	Yes	3
J.N.	F	12	20,000	Yes	2
M.F.	F	38	<10,000	Yes	1
M.F.	F	38	250,000	Yes	10
W.W.	M	70	300,000	Yes	10

\* On steroids



Table 3

## STUDIES IN PATIENTS WITH HYPERSPLENISM

Patient	Sex	Age	Diagnosis	Platelet count/cu. mm.	Platelet lifespan (days)
A.C.	F	60	Reticulum cell sarcoma	100,000	10
E.C.	F	58	Lymphosarcoma	50,000	5
G.D.	M	55	Hodgkin's	30,000	5
M.T.	F	36	Polymyositis	60,000	8
J.W.	F	68	Lymphosarcoma	50,000	10
M.N.	F	60	Felty's syndrome	100,000	11
E.S.	F	64	Cirrhosis of liver	80,000	11
J.M.	M	63	Cirrhosis of liver	100,000	10
J.T.	F	61	Cirrhosis of liver	80,000	9
J.S.	M	59	Cirrhosis of liver	110,000	9
H.M.	F	64	Cirrhosis of liver	70,000	8
A.G.	F	60	Cirrhosis of liver	90,000	10
J.C.	M	69	Cirrhosis of liver	75,000	9
B.C.	F	71	Cirrhosis of liver	70,000	10
G.S.	M	26	Spherocytosis	120,000	10
Kat	M	31	Idiopathic tropical splenomegaly	115,000	10
Kas	M	36	Idiopathic tropical splenomegaly	129,000	11
Lok	M	21	Kala-Azar	75,000	9

Table 4

CLASSIFICATION OF THROMBOCYTOPENIA

1. Shortened Survival: ITP  
    Iso-immunisation  
    Some lymphoproliferative disorders
2. Diminished Production: Marrow aplasia  
    ? Some cases of ITP
3. Sequestration and Normal Survival: Hypersplenism: Infiltrative splenomegaly  
    Congestive splenomegaly  
    Tropical splenomegaly  
    Miscellaneous splenomegaly



Effect of Incubation Time on the Uptake of  
Sodium Chromate by Platelets.

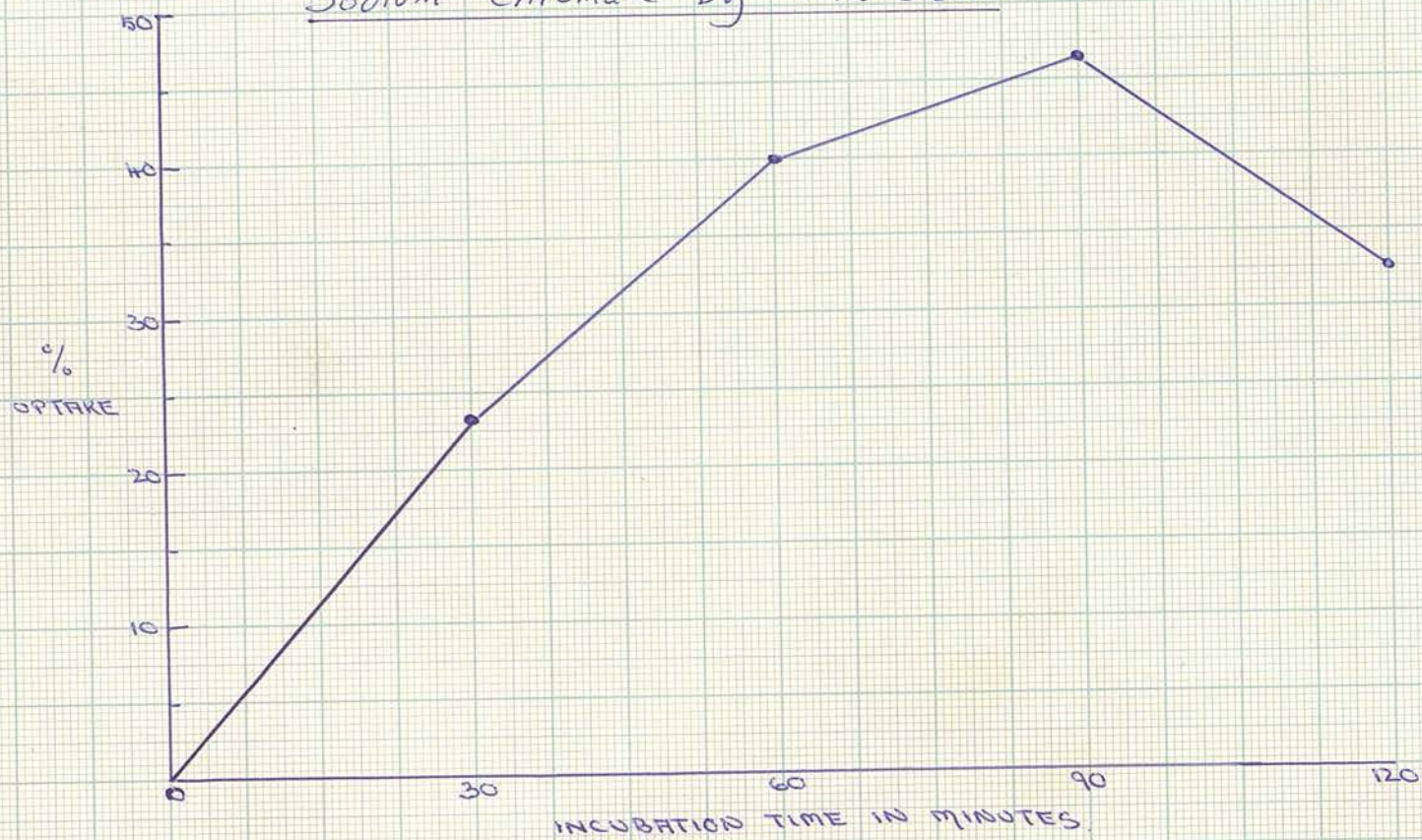


Fig. 1



Effect of Incubation Temperature on the Uptake of Sodium Chromate by Platelets

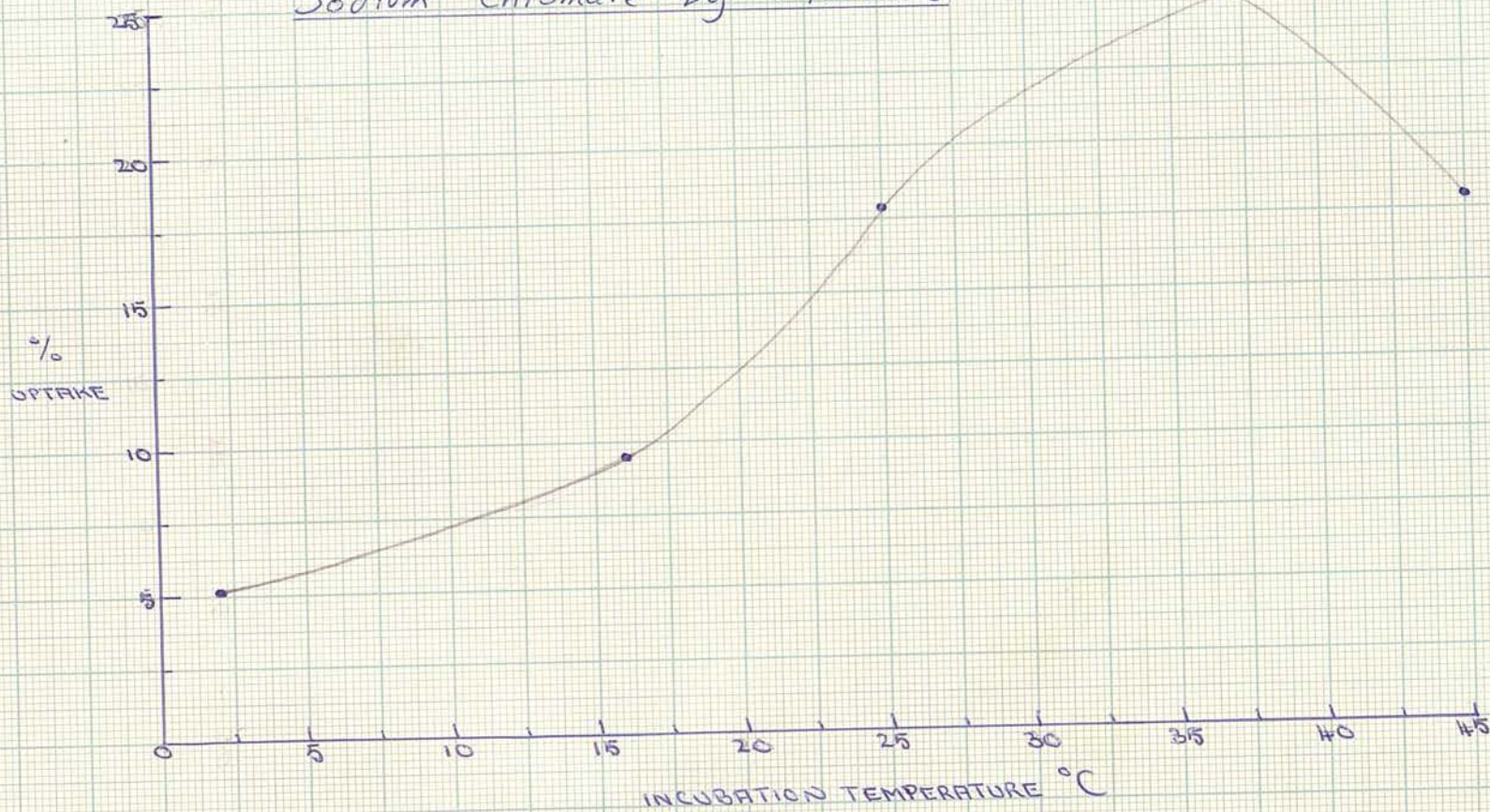


Fig. 2



Effect of Chemical Concentration of Chromium on  
the Uptake of Sodium Chromate by Platelets

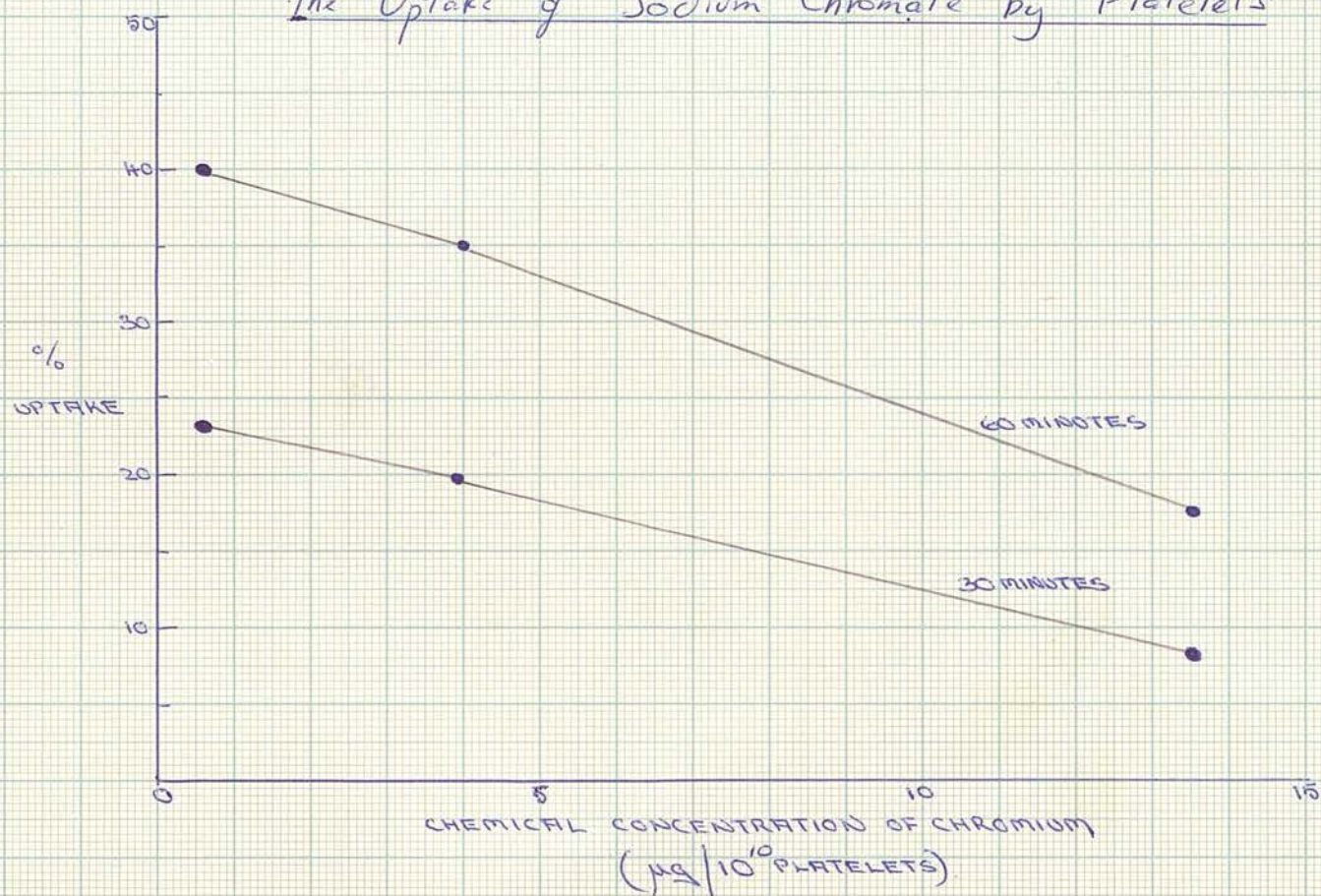


Fig 3



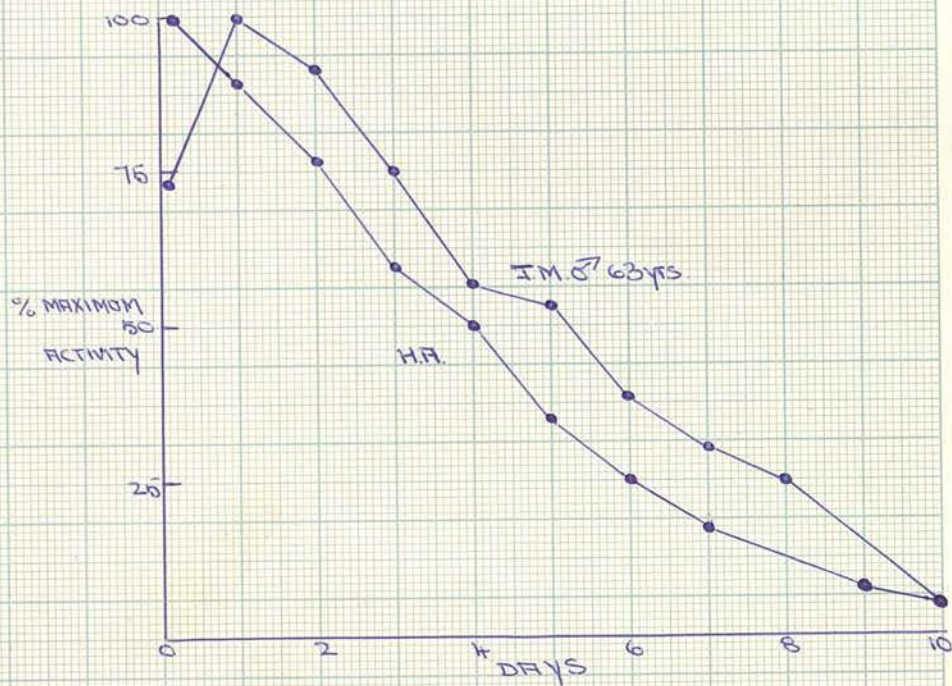
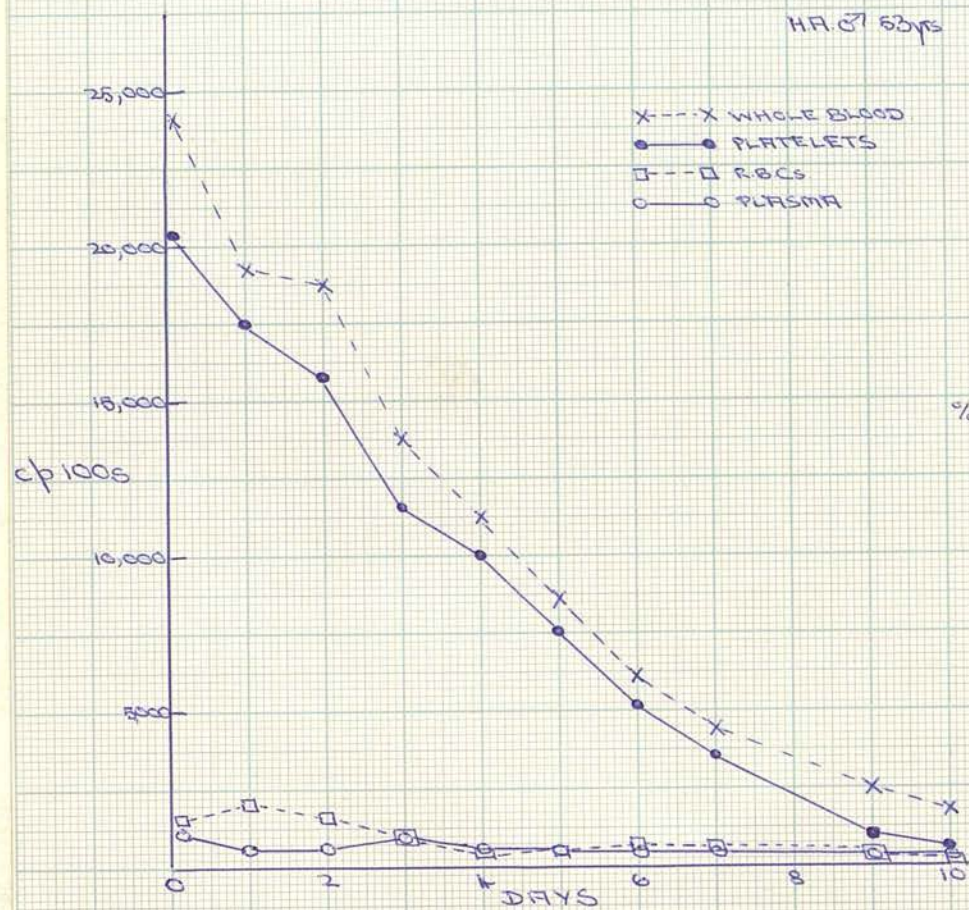


Fig. 5 - Explanation in Text.



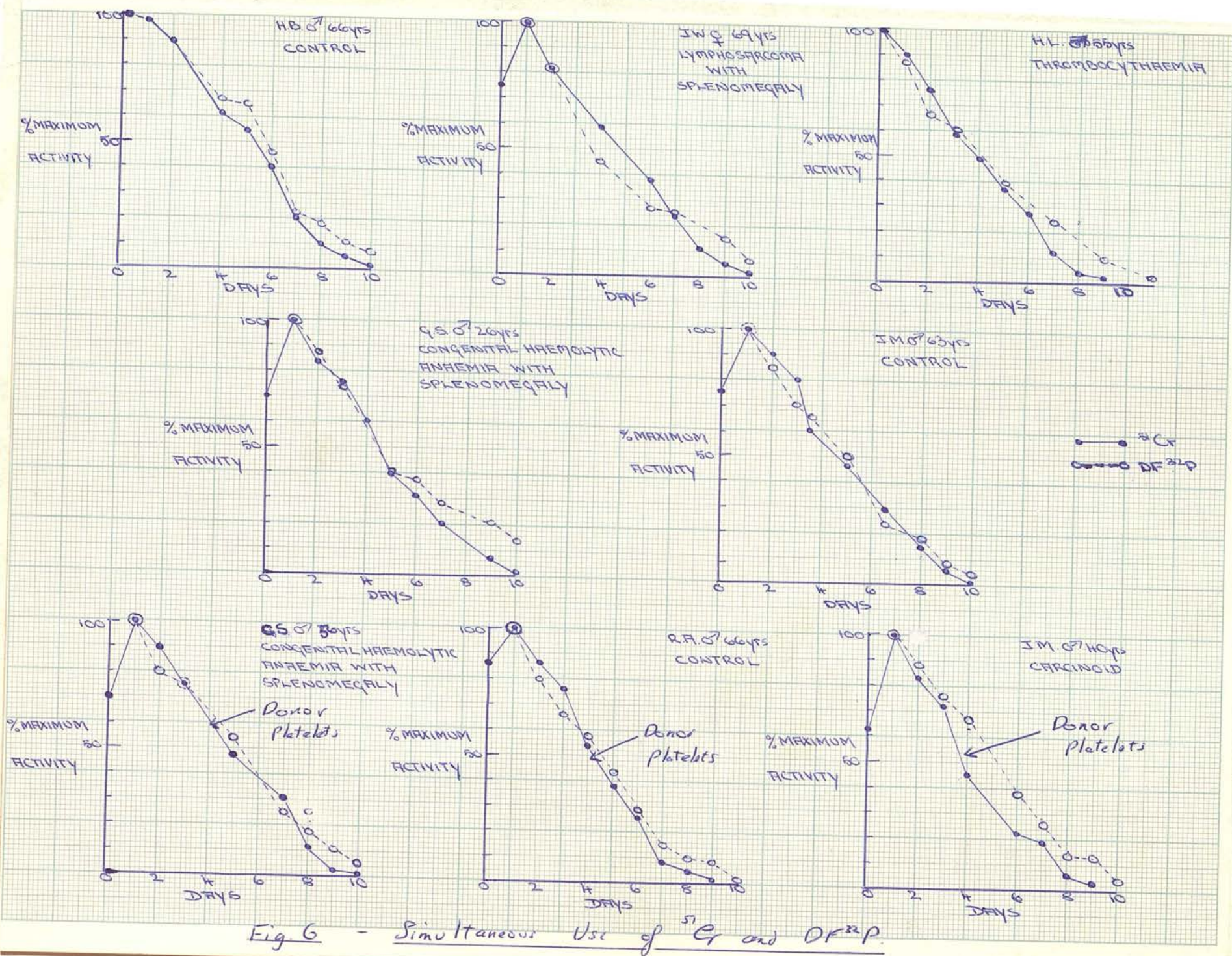


Fig 6 - Simultaneous Use of  $^{51}\text{Cr}$  and  $\text{DF}^{32}\text{P}$



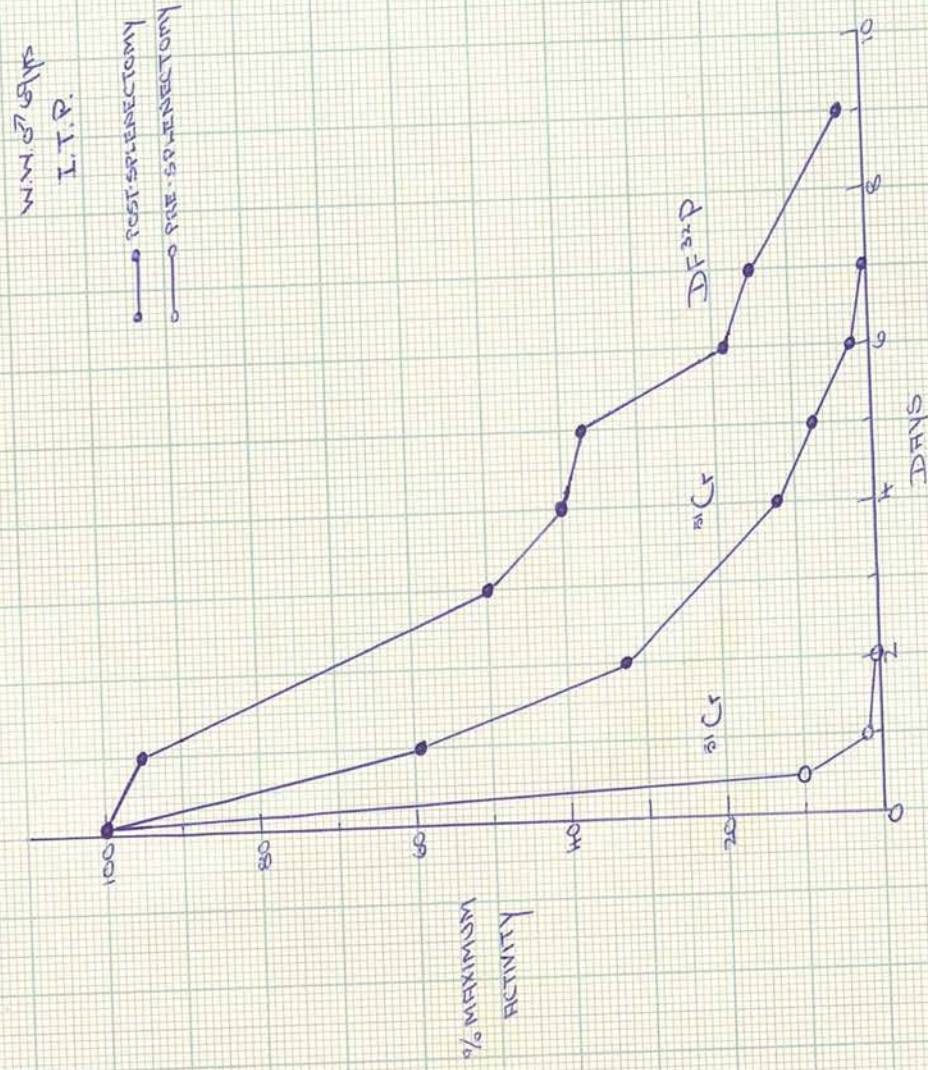


Fig 7 - Explanation in Text.



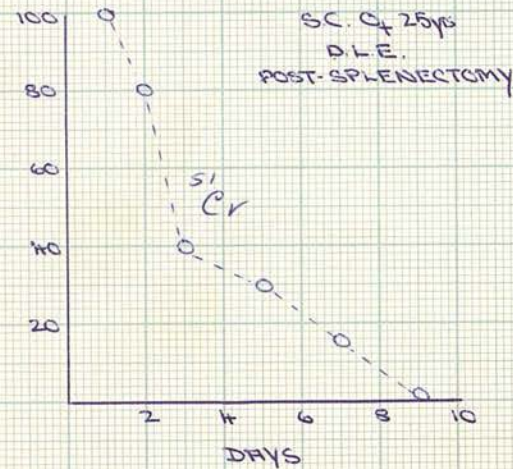
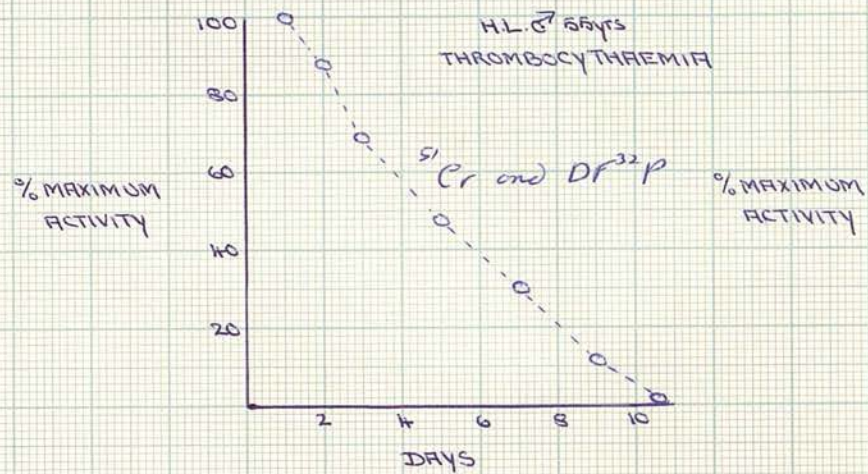
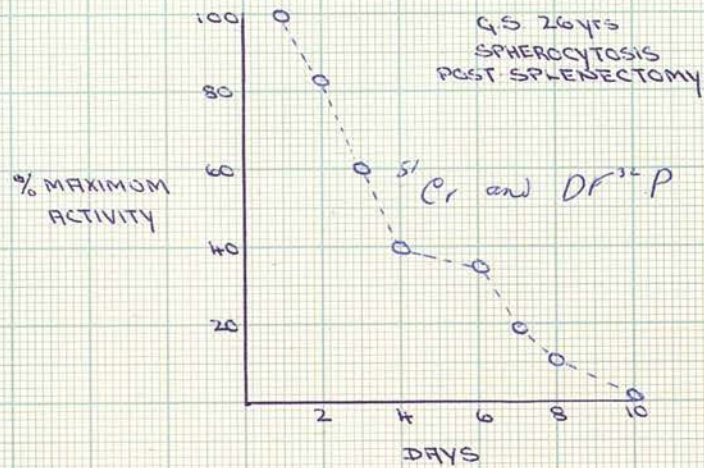
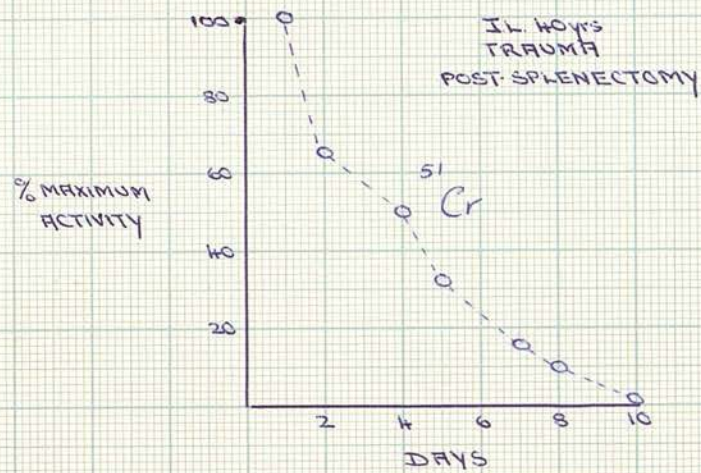


Fig 8 - Platelet Survival in Patients with Increased Platelet Counts.



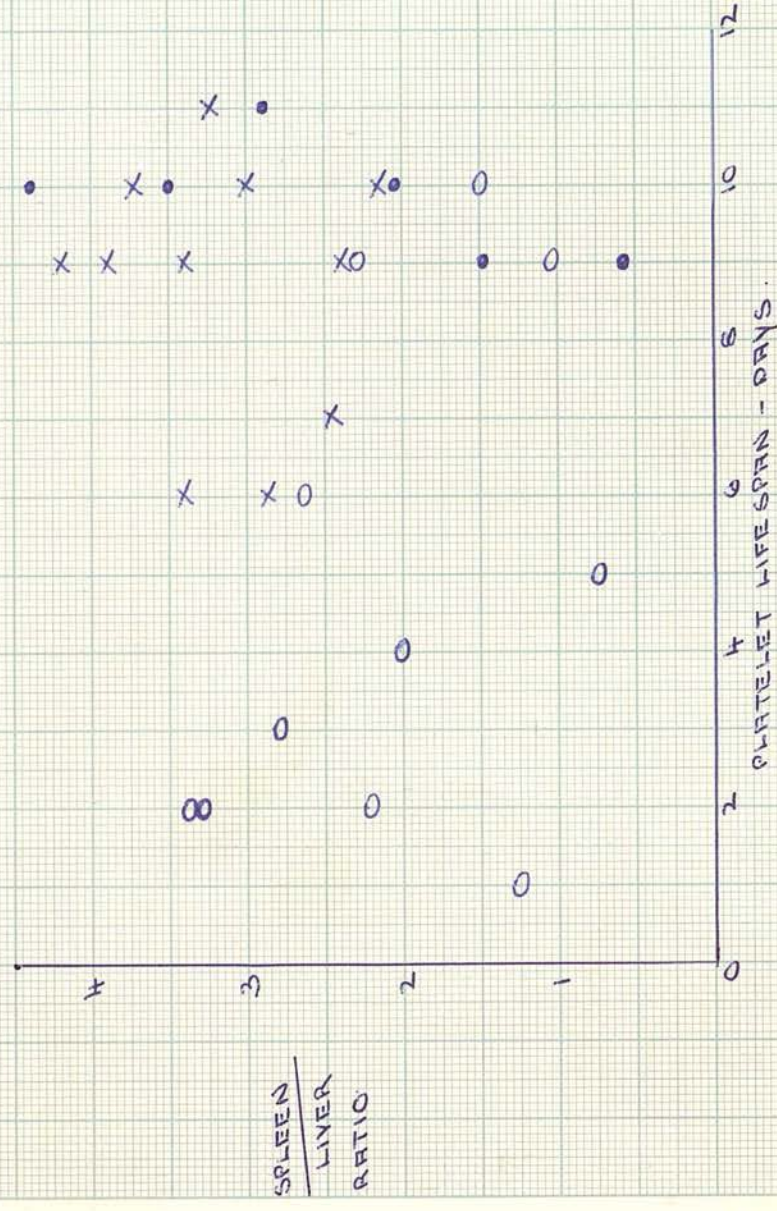
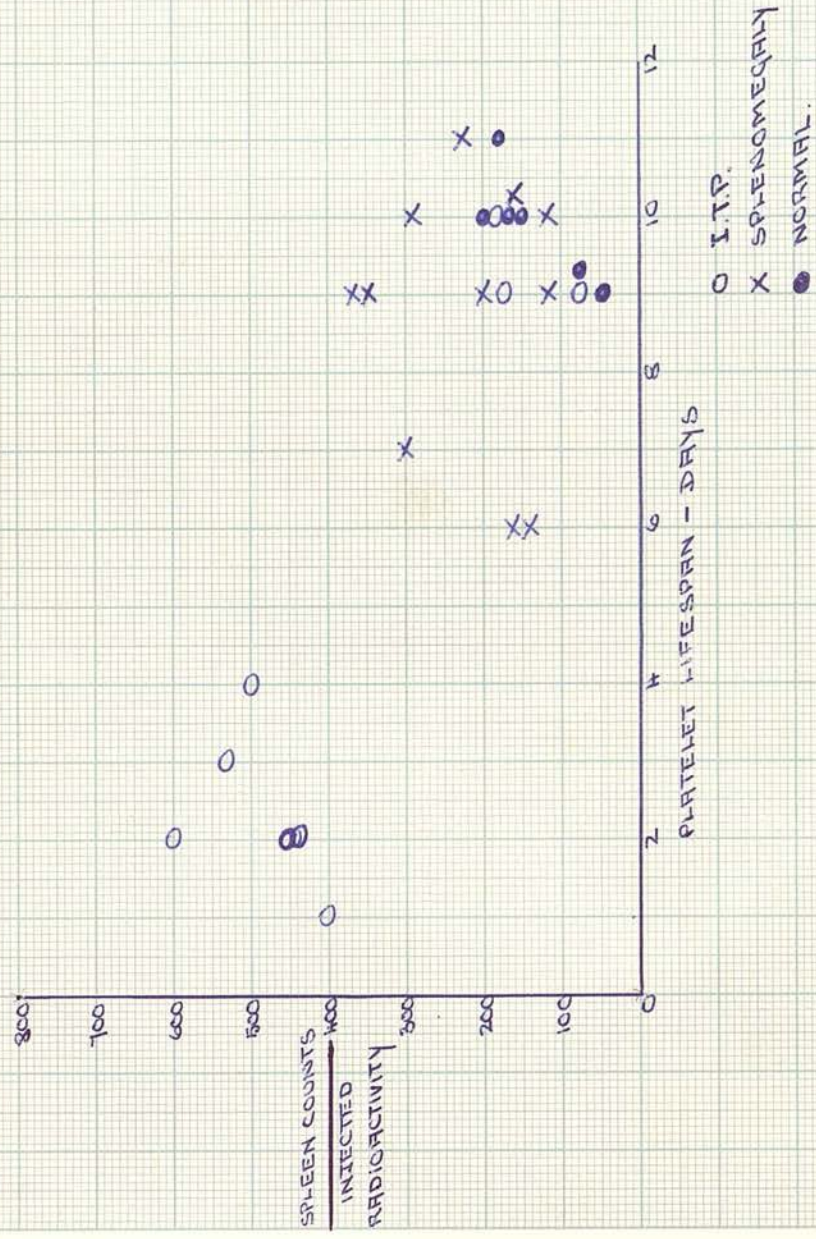


Fig. 9 - Surface Counting - Expiration in Text.



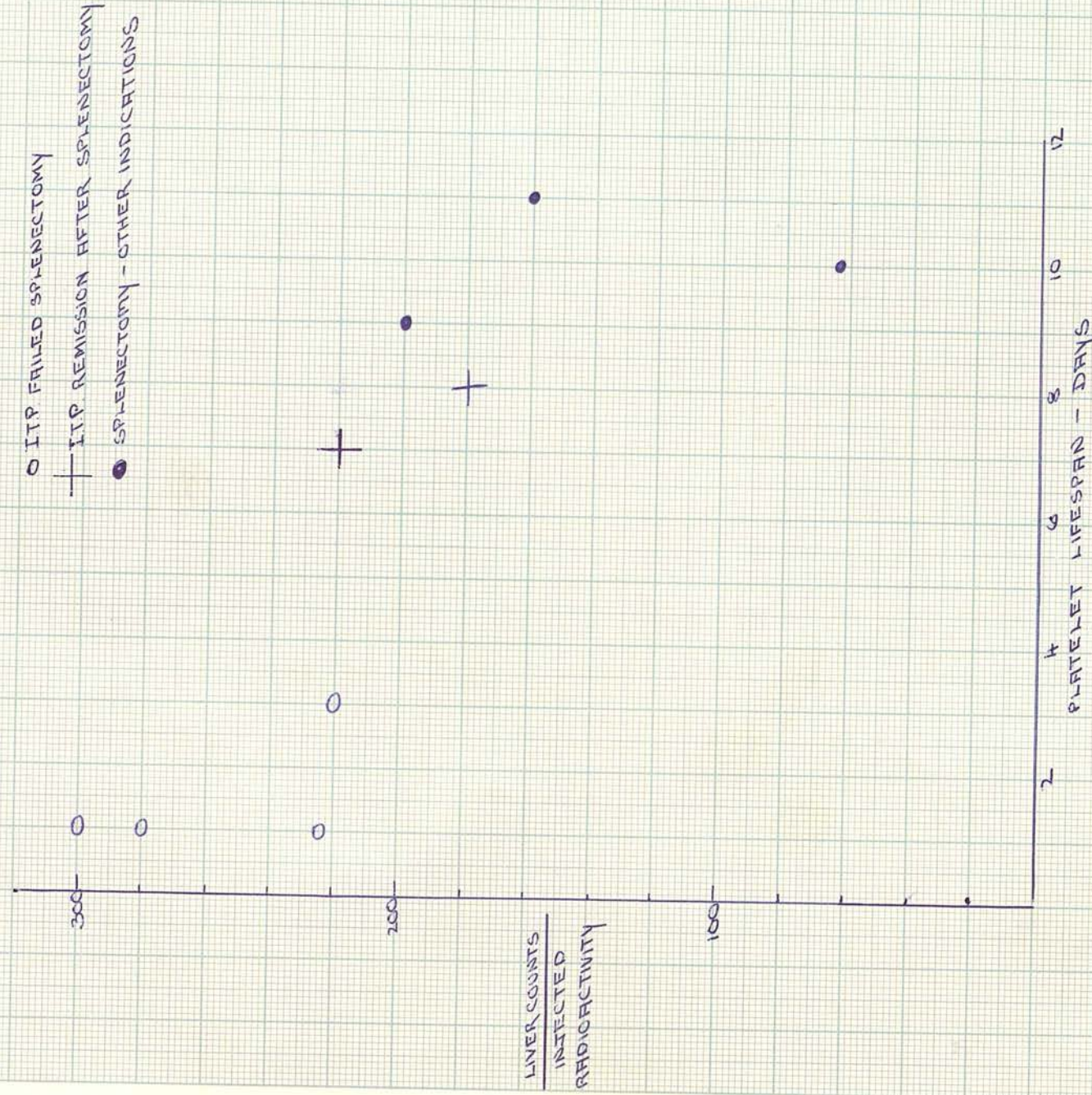


Fig 10 - Surface Counting in Patients  
after Splenectomy.